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(57) Abstract

Oligosaccharides having a high specific binding affinity for HGF growth factors and made up of relatively low molecular weight sulphated oligosaccharide chains are disclosed. The chains are composed of at least three disaccharide units including one or more internal sequences of an N-sulphated D-glucosamine 6-sulphate residue and an L-iduronic acid residue. A method is also disclosed for preparing these oligosaccharides in a purified and relatively homogeneous state from heparan sulphate. For the best HGF-binding affinity there are preferably at least five disaccharide units. The most favoured structures contain twelve or fourteen monosaccharide residues in all and include a relatively high proportion of 6-0-sulphated hexosamines, e.g. more than 30 % or even 50 %, as compared to oligosaccharide chains of unmodified native heparan sulphate. These oligosaccharides can modulate HGF activity, and uses thereof as drugs for therapeutic purposes in medicine are also disclosed.

HEPARAN SULPHATE OLIGOSACCHARIDES HAVING HEPATOCYTE GROWTH FACTOR BINDING AFFINITY

The present invention relates to certain novel oligosaccharide products and preparations thereof, useful in the field of biochemistry and medicine, which have particular binding affinity for certain growth factors or cytokines, in particular hepatocyte growth factor (HGF).

BACKGROUND

Various growth factors, often structurally unrelated, are characterised by a strong affinity for heparin. One such growth factor is hepatocyte growth factor (HGF), also known as "scatter factor". HGF is an unusually large (82kDa) and structurally complex growth factor that is synthesised as a biologically inactive single chain precursor. This is then proteolytically cleaved at a single site between linked cysteine residues giving rise to a disulphide-bonded heterodimer comprising a large α-chain (54kDa) containing a hairpin loop close to the N-terminus and a sequence of four Kringle domains, together with a smaller β-chain (26kDa). HGF is produced by various cells including fibroblasts, smooth muscle cells, kidney mesangial cells and liver non-parenchymal cells. Its target cells are primarily epithelial cells, although it also acts on endothelial cells, hepatocytes and melanocytes. It is believed to play an important role as a paracrine mediator of epithelial-mesenchymal interactions. Cellular responses, however, to HGF are complex and, as well as being mitogenic, it can also stimulate cell migration and morphogenesis depending on the cellular target and its milieu. Interestingly, it can have an anti-proliferative effect on some tumour cells, including hepatoma cells, *in vitro*. It is likely that HGF is an important factor in embryonic organ development. In the adult it has been demonstrated to have a major role in the regeneration of damaged organs, such as liver and kidney.

The cellular signal response to HGF appears to be mediated by binding (probably through the N-terminal part of the α-chain) to a single high affinity (K_d about 25pM) tyrosine kinase receptor, the product of the c-Met proto-oncogene. However, it has been demonstrated, at least with cultured cells, that a much larger number of lower affinity HGF binding sites (K_d about 350-400pM) also exist on the cell surface. It is thought possible that these lower affinity binding sites involve cell surface heparan sulphate proteoglycans, and that HGF interacts with the heparan sulphate (HS) component of such proteoglycans to cause a conformational change leading to a modulation of HGF's interaction with the c-Met receptor protein, in a way that might be analogous to the known dependence of the bFGF-receptor interaction on prior activation of the bFGF growth factor by heparan sulphate.

The present invention is based on studies in which we have shown that HGF does in fact interact in vitro with heparan sulphate. This has led to the isolation and at least partial characterisation of novel heparan sulphate oligosaccharides which exhibit significant binding affinity for HGF and which have certain structural features that contrast with those of other known growth factor binding oligosaccharides.

ABBREVIATIONS

Throughout the present specification the following abbreviations are used:

HS	- heparan sulphate;
HSPG	- heparan sulphate proteoglycan;
HGF	- hepatocyte growth factor;
dp	- degree of polymerisation (e.g. for a disaccharide, dp=2, etc);
GLcA	- β-D-glucuronic acid (or glucuronate);
IdoA	- α-L-iduronic acid (or iduronate);

IdoA(2S) - α-L-iduronic acid 2-sulphate (or iduronate);
GlcNAc - N-acetyl α-D-glucosamine;
GlcNAc(6S) - N-acetyl α-D-glucosamine 6-sulphate;
GlcNSO$_3$ - N-sulphated α-D-glucosamine;
GlcNSO$_3$(6S)- N-sulphated α-D-glucosamine 6-sulphate;
GlcNR - α-D-glucosamine with unspecified N-substituent;
∩UA - unsaturated uronic acid residue (e.g. ∩Glc for unsaturated D-glucuronic acid and ∩Hex A for unsaturated unspecified hexuronic acid residue);
SAX - strong-anion exchange;
HPLC - high performance liquid chromatography

The symbols (±2S) and (±6S) are used to denote, respectively, that a residue may or may not be sulphated at the C2 or C6 position.

SUMMARY OF THE INVENTION.

The invention provides novel oligosaccharides or preparations thereof which have a specific binding affinity for HGF. Such oligosaccharides will generally be in the form of substantially homogenous preparations consisting of oligosaccharide chains composed of a sequence of at least three disaccharide units (dp$\geq$6), preferably at least five disaccharide units (dp$\geq$10), and including a plurality of disaccharide units which each contain an IdoA(±2S) and a GlcNSO$_3$(±6S) residue and which preferably are arranged in between the terminal sugar residues of the oligosaccharide chains but not necessarily contiguously. In preferred embodiments, the oligosaccharide chains will generally also be resistant to further depolymerisation by heparinase III (heparitinase - EC 4.2.2.8), and will be obtained from heparan sulphate or from other natural heparan type material.

More specifically, in one aspect the invention

consists in an oligosaccharide preparation obtainable from partially depolymerised heparan sulphate (HS) or other natural heparin type material as a fraction thereof, characterised in that the oligosaccharide preparation consists essentially of oligosaccharide chains which have a specific binding affinity for hepatocyte growth factor (HGF) and which are composed of a sequence of at least three disaccharide units ($dp \geq 6$) that includes at least two disaccharide units containing an L-iduronic acid residue IdoA(±2S) and an N-sulphated D-glucosamine residue $GlcNSO_3$(±6S).

In another aspect the invention can alternatively be defined as an oligosaccharide preparation comprising heparan sulphate (HS) fragments which have a specific binding affinity for hepatocyte growth factor (HGF) and which are composed of oligosaccharide chains containing a sequence of at least three disaccharide units ($dp \geq 6$) that includes at least two disaccharide units containing an L-iduronic acid residue IdoA(±2S) and an N-sulphated D-glucosamine residue $GlcNSO_3$(±6S).

Preferably, one or more of the above-mentioned at least two disaccharide units in the oligosaccharide chains is IdoA-α1,4-$GlcNSO_3$(6S), and the preparations may be such that the HGF-binding affinity is not completely destroyed by treatment under depolymerising conditions with heparinase I. At least the majority of the oligosaccharide chains may have substantially the same length as a result of carrying out a size fractionation separation procedure, and in preferred embodiments at least the majority of the oligosaccharide chains each have a degree of polymerisation (dp) of 10 or more, but with the maximum size being no greater than ten disaccharide units in total. More preferably, the oligosaccharide chains each have a degree of polymerisation (dp) of 12 or 14.

As already indicated, in preferred embodiments the

oligosaccharide chains of the preparations of this invention are substantially completely resistant to further depolymerisation upon treatment under enzymic depolymerising conditions with heparinase III (heparitinase I). Also, the IdoA(2S) content, if any, of said oligosaccharide chains will be less than the unsulphated IdoA content thereof, and in general the oligosaccharide chains will usually contain a relatively high proportion of 6-O-sulphated hexosamines compared to oligosaccharide chains of unmodified native heparan sulphate. Thus, it may be expected that the content of glucosamine residues in the oligosaccharide chains which are O-sulphated at C6 will usually be greater than 24%, for example about 35% or greater. More specifically, the $GlcNSO_3(6S)$ content of the oligosaccharide chains, i.e. number of residues per 100 disaccharides, is preferably at least 30% and may be 50% or more.

In at least most embodiments the structure of the oligosaccharide chains will include internal repeat sequences of IdoA(±2S) and $GlcNSO_3(6S)$ interrupted by occasional GlcNAc(±6S) components, and in presently preferred embodiments substantially all said oligosaccharide chains consist of a sequence of six or seven disaccharide units in all.

Oligosaccharide preparations of this invention will generally be obtainable by enzymic partial depolymerisation to the fullest extent of heparan sulphate using heparinase III (heparitinase I), followed by size fractionation using, for example, gel filtration size exclusion chromatography, and then, in respect of a selected fraction or fractions recovered from the size fractionating stage, affinity chromatography using an HGF growth factor as the immobilised ligand in order to separate out HGF-binding fragments, and then eluting selectively over a range of salt concentrations under a salt gradient to fractionate said fragments in respect of

HGF binding affinity, followed by recovering the most strongly bound fragments and, optionally, further purifying the recovered product by carrying out at least one additional step of size fractionation and selection of recovered product. The heparan sulphate (HS) may be derived from human fibroblast heparan sulphate proteoglycan (HSPG) or any other suitable biological source.

The invention may also be defined as providing an oligosaccharide preparation made up of oligosaccharide chains having a specific binding affinity for human hepatocyte growth factor (HGF), characterised in that

(a) it is composed predominantly of a molecular species:

$$X-\left[-Y-\right]_n-Z$$

in which

X is ∆HexA-$GlcNSO_3$

Y is IdoA(±2S)-GlcR(±6S),

Z is IdoA-GlcR

where R is NSO_3 or NAc, and

n is in the range 1 to 5

with the proviso that when n is three or more then at least for the majority of said molecular species two or more of the $GlcR_3$ residues in Y are N-sulphated glycosamines sulphated at C-6, i.e. $GlcNSO_3$(6S); and

(b) it is obtainable by a process comprising the steps of digesting a heparan sulphate with heparinase III (heparitinase I) so as to bring about partial depolymerisation thereof to the fullest extent, followed by size fractionating the oligosaccharide mixture produced using for example gel filtration size exclusion chromatography, collecting a fraction or fractions containing oligosaccharide chains

having a particular size selected within the range of 10 to 20 monosaccharide residues, then subjecting said selected fraction or fractions to affinity chromatography using an immobilised HGF ligand and recovering the more strongly HGF-binding constituents by eluting under a salt gradient over a range of salt concentrations and collecting a selected fraction or fractions containing the bound material which desorbs only at the highest salt concentrations.

In at least most preferred embodiments the symbol Y in the above structural formula will represent primarily or exclusively IdoA-GlcNSO$_3$(±6S), and n is the range 3 to 5, preferably 4 or 5 so that said molecular species consists of a total of six or seven disaccharide units in all. In addition, the content of glucosamine residues having a 6-O-sulphate group will be greater than 24%. Usually, the content of IdoA(2S), if any, will be small in these embodiments.

The invention also provides an oligosaccharide preparation having a specific binding affinity for hepatocyte growth factors (HGF's) that is substantially wholly composed of oligosaccharide chains which are twelve or fourteen monosaccharide residues in length and which contain an internal sequence comprising at least 2 disaccharide units each consisting of an IdoA residue linked to a GlcNSO$_3$(±6S) residue, with more than 20% of the glucosamine residues (terminal or internal) being 6-O-sulphated. In accordance with this aspect of the invention, substantially all the oligosaccharide chains may have the following sequence

$$\Delta GlcA\text{-}GlcNSO_3\text{-}[IdoA\text{-}GlcNSO_3(\pm 6S)]_n\text{-}IdoA\text{-}GlcR$$

where R is NSO$_3$ or NAc, and n is 4 or 5.

In a further aspect, the present invention embraces a

method or process for obtaining oligosaccharides that have a particular binding affinity for hepatocyte growth factor, characterised in that partial depolymerisation products of heparan sulphate, produced by treatment with a selective scission reagent that cleaves the polysaccharide chains thereof selectively in regions of relatively low sulphation, are subjected to affinity chromatography using HGF as the immobilised ligand so as to separate out HGF-binding fragments, the more strongly binding constituents then being recovered by eluting under a salt gradient and collecting a selected fraction or fractions containing the bound material which desorbs at the higher salt concentrations. More specifically from this aspect the invention provides a method of isolating from heparan sulphate derived from heparan sulphate proteoglycan of mammalian cells low molecular weight oligosaccharides in a purified and relatively homogeneous state which have a specific binding affinity for hepatocyte growth factor, said method comprising the steps of

(a) preparing an affinity chromatographic matrix or substrate incorporating a sample of hepatocyte growth factor (HGF) as the affinity ligand immobilised thereon;

(b) treating said heparan sulphate with a selective scission reagent so as to cleave the polysaccharide chains thereof selectively in regions of relatively low sulphation;

(c) subjecting the product of step (b) to size fractionation, for example by gel filtration size exclusion chromatography, and collecting selectively therefrom fractions that appear to contain oligosaccharides composed of less than ten disaccharide units,

(d) contacting the affinity chromatographic matrix or substrate from step (a) with a selected fraction, or set of fractions, from step (c) containing a specific number of disaccharide units in the range of five to seven in order to

extract from the latter and retain on said matrix or substrate size selected oligo-saccharide fragments of the heparan sulphate glycosaminoglycan that have at least some binding affinity for the immobilised HGF;

(e) eluting the affinity chromatographic matrix or substrate using a progressively increasing salt concentration or gradient in the eluant;

(f) collecting the fraction or set of fractions containing oligosaccharide fragments eluting in selected highest ranges of eluant salt concentration; and optionally,

(g) further purifying the product of the selected fraction, or set of fractions, from step (f) by selectively repeating step (c) using said selected fraction or set of fractions collected in step (f) instead of the reaction mixture obtained from step (b), and optionally also repeating steps (d), (e) and (f).

In carrying out the above-specified method it will be appreciated that preferably the selective scission reagent is heparinase III (heparitinase I) and the heparan sulphate is partially depolymerised to the fullest extent by digesting therewith until cleavage of the heparitase III sensitive linkages is complete. Also, the fractions collected from the size fractionation stage will preferably be those that appear to contain oligo-saccharides composed of six or seven disaccharide units.

The oligosaccharide preparations are applicable for therapeutic use, acting as an HGF-activity modulating agent for controlling or reducing cell growth, proliferation or migration in treating mammals in need of such treatment. Thus, the invention also provides pharmaceutical formulations or compositions for medical use comprising a therapeutically effective non-toxic amount of an HGF-activity modulating agent comprising an

oligosaccharide preparation as herein specified, or pharmaceutically acceptable salts thereof, together with a pharmaceutically acceptable carrier or vehicle.

A pharmaceutical composition or formulation in accordance with the invention for use in controlling the activity of hepatocyte growth factors in mammals may also be defined as comprising a therapeutically useful amount of an essentially pure oligosaccharide preparation having a specific binding affinity for hepatocyte growth factors (HGF's), consisting essentially of linear oligosaccharide chains which are substantially homogeneous with respect to HGF binding affinity and which contain a sequence of less than ten disaccharide units including, intermediate its terminal residues, a plurality of disaccharide units each composed of an N-sulphated glucosamine residue (±6S) and an unsulphated iduronic acid residue.

The invention will be further described, with reference to the accompanying drawings, in relation to some of the background experimental work carried out by the inventors which brings out various further features of the invention and illustrates the way in which HGF-binding oligosaccharides in accordance with the invention may be isolated and characterised. Accordingly, from this description the skilled person in the art will more readily be able to appreciate the nature of the invention and will more readily be able to put it into practical effect.

BRIEF DESCRIPTION OF THE DRAWINGS:

FIGURE 1: This shows the comparative affinities of ^{3}H-heparin (panel A), $^{3}H^{35}S$-liver HSPG (panel B) and $^{3}H^{35}S$-liver HS chains (panel C) for an HGF affinity column, samples being applied in 0.15M NaCl and eluted with a step gradient of 0.2 - 1.0M NaCl as shown by the arrows in panel A.

FIGURE 2: This shows the effect of various specific modifications or depolymerisations of fibroblast HS on its affinity for HGF. Samples of ^{3}H-labelled fibroblast HS, intact (panel A) or after low pH nitrous acid degradation (panel B), or after solvolytic de-N-sulphation/re-N-acetylation (panel C), or after heparinase III digestion (panel D), or after heparinase I digestion (panel E), were applied to the HGF affinity column in 0.15M NaCl. Bound material was then eluted with a step gradient of 0.2 - 1.0M NaCl as shown by the arrows in panel A.

FIGURE 3: This shows a size fractionation of heparinase III-resistant oligosaccharides. ^{3}H-fibroblast HS was exhaustively digested with heparinase III and the digest was fractionated into its constituent oligosaccharide sizes by gel filtration chromatography on Bio-Gel P10. Oligosaccharide fractions corresponding to dp2 - dp12/14 (where dp is the number of monosaccharide units) were individually recovered.

FIGURE 4: This shows the effect of HS oligosaccharide size on HGF affinity. ^{3}H-Fibroblast HS was digested with heparinase III and size fractionated on a Bio-Gel P10 column. Fractions corresponding to oligosaccharide sizes of dp6 (panel B), dp8 (panel C), dp10 (panel D) and combined dp12/14 (panel E) were tested for affinity to HGF and compared with the affinity of intact parent HS (panel A). Samples were applied in 0.15M NaCl and bound material was step eluted with increasing 0.2 - 1.0M NaCl concentrations as shown by the arrows in panel A.

MORE DETAILED DESCRIPTION

Background experimental work was carried out using, as source materials, recombinant human HGF which was purified from the culture medium of cells transfected with a plasmid containing the human HGF cDNA (see Nakamura *et al*, (1989) *Nature* 342, 440-443), and HSPG which was

prepared from both (a) culture medium from confluent cultures of a human foetal skin fibroblast cell line biosynthetically radiolabelled with ^{3}H-glucosamine (see Turnbull, J.E. and Gallagher, J.T. (1991), Biochem, J. 272, 553-559) and (b) rat livers biosynthetically radiolabelled *in vivo* with ^{3}H-glucosamine and $Na_2{}^{35}SO_4$ (see Lyon, M. and Gallagher, J.T. (1991), Biochem, J. 273, 415-422. From the HSPG, radiolabelled heparan sulphate (HS) chains were prepared by exhaustive proteolytic digestion with Pronase. Thus, in an example of one particular procedure HS chains were obtained from cultured foetal skin fibroblasts grown in MEM containing 10% (v/v) heat-inactivated donor calf serum (Gibco) and 1mM glutamine. Confluent cultures were metabolically radiolabelled with 10μCi/ml of D-[6-^{3}H]-glucosamine hydrochloride for 72 hours. The culture medium was removed and kept to one side whilst the cell layers were extracted with 0.15M NaCl, 20mM sodium phosphate, 1% (v/v) Triton X-100 pH 7.0 for 1 hour at room temperature with agitation. The cell layer extracts were recombined with the culture supernatants and the whole was digested with Pronase (100μg/ml) for 3 hours at 37°C. The digest was heated to 100°C for 5 minutes, clarified by centrifugation and then applied to a small DEAE-Sephacel column. This was washed extensively with 0.3M NaCl, 20mM sodium phosphate, 1% (v/v) Triton X-100 pH 7.0 after which the ^{3}H-labelled sulphated GAGs were recovered by step elution with 1.5M NaCl, 20mM sodium phosphate, 1% (v/v) Triton X-100, pH 7.0. The recovered material (comprising mixed HS and CS/DS) was dialysed against 50mM NaCl, 50mM Tris HCl, pH 8.0, concentrated to approximately 1ml by reverse osmosis against poly(ethylene glycol) and then digested with 0.1unit/ml of chondroitinase ABC for 4 hours at 37°C. The intact HS chains were recovered by re-application of the digest to a small DEAE-Sephacel column, which was eluted as described above but omitting the Triton X-100. The ^{3}H-HS chains were precipitated from the 1.5M NaCl eluant by

addition of 3 vols of 95% (v/v) ethanol, air-dried and re-dissolved in distilled water.

The HS chains were selectively depolymerised either with heparinases or low pH nitrous acid, using methods performed essentially as described in Turnbull and Gallagher (Biochem, J. (1991), 273, 553-559). Solvolytic N-desulphation of fibroblast HS, followed by re-N-acetylation with acetic anhydride was also performed using the method of Inoue and Nagasawa (Carbohydr. Res. (1976), 46, 87-95). The content of the above-mentioned papers are incorporated herein by reference.

Heparinase Enzymes

The polysaccharide lyase enzyme heparinase I (Flavobacterium heparinum; EC 4.2.2.7) referred to herein was supplied by Seikagaku Kogyo Co., Tokyo, Japan, but heparinase II (F. heparinum; no EC number assigned) and heparinase III (F. heparinium; EC 4.2.2.8) were from Grampian Enzymes of Aberdeen, Scotland. Heparinase III is in fact substantially the same as the enzyme supplied under the designation heparitinase I by Seikagaku Kogyo Co. Heparinase III (heparitinase I) will selectively cleave glycosidic linkages on the non-reducing side of GlcA-containing disaccharides, such as in GlcNAc-α1,4-GlcA present in regions of low sulphation, but in general it will not cleave bonds of sulphated disaccharides containing L-iduronic acid or 2-sulphated L-iduronic acid, i.e. IdoA or IdoA(2S). This is in contrast to the enzyme heparinase I (EC 4.2.2.7) which cleaves glycosidic linkages of disaccharides containing 2-sulphated L-iduronic acid. For a review of these enzymes see R J Linhardt *et al* (1990) *Biochemistry* 29, 2611-2617.

In connection with the cleavage of polysaccharide or oligosaccharide glycosidic linkages, e.g. 1,4 linkages, by enzymes such as heparinase I and heparinase III, it should

incidentally be appreciated that in one of the fragments produced the monosaccharide residue at the non-reducing end which is immediately adjacent the cleaved bond will generally become unsaturated with a double-bond formed between C4 and C5. This unsaturation, however, is not likely to affect significantly the growth factor binding affinity of the fragment concerned, although it may perhaps affect stability of the molecule.

HGF - affinity chromatography

In the course of this work, HGF binding affinity of the HSPG, HS and oligosaccharide HS depolymerisation products was investigated using affinity chromatography with an HGF-affinity matrix or substrate, from which the HGF binding constituents were eluted and selectively recovered using a salt gradient.

For preparing the HGF affinity matrix, Affi-Gel 10 (RTM) activated affinity gel (from Bio-Rad Laboratories) was washed following the supplier's instructions. A portion of the recombinant human HGF (100µg) was pre-mixed with an excess of heparin (500µg) in 100µl of coupling buffer (0.1M HEPES, 80mM NaCl, pH 7.0) and incubated for 20 minutes at room temperature. The mixture was then added to 300µl of the washed Affi-Gel 10 and the volume adjusted to 1ml with the coupling buffer. This was mixed end-over-end for 10 minutes at room temperature before the addition of 0.5ml of 1M ethanolamine to block remaining active groups on the gel. After a further 1 hour of mixing the gel was transferred to a small column, washed extensively with 1.5M NaCl, 20mM sodium phosphate pH 7.0, and then re-equilibrated in 0.15M NaCl, 20mM sodium phosphate, 0.2mM sodium azide pH 7.0. When not in use the column was stored in this solution at 4°C. A control column was also prepared exactly as described above, but omitting the HGF.

In performing the affinity chromatography, the radiolabelled samples, diluted where necessary to an ionic strength ≤0.15M NaCl, were each applied to the column and recirculated a number of times e.g. at a flow rate of 0.5ml/min. and at room temperature, so as to maximise opportunity to bind to the HGF. The column was then washed with 5ml of 0.15M NaCl, 20mM sodium phosphate pH 7.0, followed sequentially with 5ml volumes of 0.2, 0.4, 0.6, 0.8 and 1.0M NaCl in 20mM sodium phosphate pH 7.0. When liver HSPG was chromatographed on the HGF column 0.1% (w/v) CHAPS was included in all the solutions. Fractions of 1ml were collected and monitored for radioactivity.

In carrying out the selective depolymerisation operations, enzymatic digestions of HS with either heparinase I or heparinase III were performed with additions of 20mIU/ml of enzyme in 0.1M sodium acetate, 0.1mM calcium acetate, 1mg bovine serum albumin/ml, pH 7.0 at 37°C. In order to ensure maximum breakdown of the HS three additions of enzyme were made over an 18 hour period. For the de-N-sulphation of HS this was carried out by solvolysis of the pyridinium salt in 95% (v/v) dimethyl sulphoxide/5% (v/v) methanol, followed by acetylation of the resulting free amine groups with acetic anhydride in accordance with the method of Inoue and Nagasawa previously referred to.

To prepare the HS oligosaccharides, ^3H fibroblast HS was degraded with heparinase III as described above. The digest was then separated into its constituent oligosaccharide size fractions by gel filtration chromatography on a Bio-Gel P10 column (1x115cm) eluted with 0.2M NH_4HCO_3 at a flow rate of 5ml/hr. The peaks corresponding to oligosaccharides from dp2 to a combined dp12/14 fraction were individually pooled and repeatedly lyophilised to remove the NH_4HCO_3.

Disaccharide composition of HS oligosaccharides

Disaccharide compositions of specific HS oligosaccharide fractions recovered from the affinity chromatography stage were analysed after exhaustive digestion and complete depolymerisation with a combination of the enzymes heparinase I, II and III. The digestion mixture was generally made up of 20mIU/ml each of heparinases I, II and III in 0.1M sodium acetate, 0.1mM calcium acetate, 1mg bovine serum albumin/ml pH 7.0 at 37°C. Three additions of enzymes were made over an 18 hour digestion period. The digest was then chromatographed on a Bio-Gel P2 column (1 x 111cm) eluted with 0.2M NH_4HCO_3 at a flow rate of 4ml/hr. Fractions corresponding to disaccharides were pooled, repeatedly lyophilised and finally re-dissolved in distilled water adjusted to pH 3.5 by the addition of HCl. Samples were then injected onto a Spherisorb (RTM) 5µm SAX (strong anion-exchange) column (Technicol, Stockport, UK) linked to a Dionex HPLC system. The column was washed with 5ml of acidified water pH 3.5 followed by elution of the constituent disaccharides with a 40ml gradient of 0-0.75M NaCl, pH 3.5 at a flow rate of 1ml/min. The eluant was monitored with an on-line Radiomatic Flo-One/Beta Series A-200 radioactivity detector (Canberra Packard) using a 0.5ml flow-through liquid cell and a scintillant:sample ratio of 3:1. The identities of the constituent disaccharides were determined by comparison with the elution positions of eight known disaccharide standards monitored by UV detection at 232nm.

Interaction of intact and partially depolymerised HS with HGF affinity column

It was found that liver HSPG bound strongly to the HGF affinity column with the majority of the bound material requiring 0.6 and 0.8M NaCl for eluting as shown in FIGURE 1B. The abundant unbound fraction would also

bind if re-applied and was due to overloading of the column. Pronase-released HS chains also bound strongly, although the proportion that eluted at the higher step (0.8M NaCl) was reduced (see FIGURE 1C). It is believed that the higher affinity of the intact HSPG may reflect the polyvalency of the HSPG and the greater possibility of bridging more than one immobilised HGF molecule. Nevertheless, the similar affinities of the HSPG and HS chains demonstrated that the affinity resides in the HS moiety with little, if any, contribution from the protein core. In comparison, commercial ^{3}H-heparin was found to bind with an apparent affinity similar to that of the intact HSPG (see FIGURE 1A).

<u>Identification of the major structural determinants for HS binding to HGF</u>

Elucidation of the principal structural determinants for interaction with HGF was undertaken by comparison of the effects of various specific chemical and enzymic modifications or depolymerisations on the ability of fibroblast HS (which binds to HGF with characteristics similar to liver HS) to bind to the HGF affinity column. Deaminitive scission with low pH nitrous acid, which specifically cleaves N-sulphated disaccharides with concomitant loss of the N-sulphate group, completely abolished binding to the column (see FIGURE 2B and compare with FIGURE 2A). This demonstrated the inability of the nitrous acid resistant, mainly non-sulphated, blocks of GlcA- GlcNAc to support interaction and suggested a requirement for N-sulphate groups. However, alternative chemical de-N-sulphation of HS by solvolysis (with replacement of the N-sulphates by N-acetyl groups), without the concomitant depolymerisation of the HS chain that occurs with nitrous acid, had relatively little effect on the HGF binding (see FIGURE 2C), elution occuring at just one step lower, i.e. 0.6M, compared to 0.8M NaCl for the native unmodified HS. This indicated

that N-sulphates *per se* make no more than a minor contribution to the binding activity and that the major binding determinants are other structural features spatially associated with the N-sulphation or $GlcNSO_3$ residues in such a way as to be similarly disrupted by nitrous acid depolymerisation treatment. Since both iduronate residues IdoA(±2S) and 6-O-sulphated hexosamines are biosynthetically linked to the presence of N-sulphate, it was deduced that these are likely to provide the major binding determinants.

Further elucidation was gained from the analysis of individual enzymic depolymerisations with heparinases I and III. Heparinase I, which specifically cleaves N-sulphated disaccharides containing IdoA(2S) residues, especially $GlcNSO_3$(±6S)-IdoA(2S), generated relatively large resistant fragments from fibroblast HS which would have internal sequences containing non-sulphated GlcA/IdoA. However, the treatment had relatively little effect on HGF binding with most material eluting at 0.4M and 0.6M NaCl (see FIGURE 2E). This indicated that interaction with HGF does not require clusters or contiguous sequences of two or more disaccharides containing IdoA(2S) residues. In comparison, digestion with heparinase III, which cleaves HS in regions of low sulphation so as to excise and depolymerise nearly all GlcA-containing disaccharides (mainly GlcNAc-GlcA) to give rise to enzyme resistant oligosaccharide sequences which are of a generally smaller size than with heparinase I (being made up primarily of contiguous sequences of $GlcNSO_3$-IdoA with variable O-sulphation), generated a more complex pattern (see FIGURE 2D) with fragments of mixed HGF affinities. Here, the majority of the material did not bind, but that which did eluted predominantly at 0.4M and 0.6M with only a small amount at 0.2M. Since Heparinase III-resistant oligosaccharides will be enriched in IdoA(±2S), in the light of previous observations including the fact that HGF binding is not particularly

sensitive to heparinase I, this result further indicates that non-sulphated IdoA and/or 6-O-sulphates are the most important determinants for HGF binding.

Analysis of HGF-binding oligosaccharides

The various HGF-binding fractions from a heparinase III digest of ^{3}H-fibroblast HS (FIGURE 2D) were analysed for their relative size distribution by gel filtration exclusion chromatography on Bio-Gel P10 (not shown). It was found that the non-binding (0.15M NaCl) and weakly bound (0.2M NaCl) fractions comprised predominantly dp2-4 oligosaccharides. In contrast, the medium (0.4M NaCl) and high (0.6M NaCl) affinity fractions contained oligosaccharides of dp6-10 and dp≥10 (mostly dp12) respectively.

Size dependence was analysed in more depth by collecting individual oligosaccharide fractions from a preparative gel filtration chromatography fractionation of a large-scale heparinase III digest of ^{3}H-fibroblast HS. The digest was fractionated on Bio-Gel P10 into oligosaccharides ranging in size from dp2 to a mixed dp12/14 fraction (see Fig. 3). These oligosaccharide fractions were individually assayed for HGF-binding activity. The general trend was for HGF affinity to increase with oligosaccharide size (see FIGURE 4). Dp2 and dp4 oligosaccharides did not bind to HGF in 0.15M NaCl (data not shown). The smallest oligosaccharides which exhibited some binding to HGF at NaCl concentrations above 0.15M NaCl were dp6 in which a small proportion of oligosaccharides eluted with 0.4M NaCl (see Fig. 4B). The majority of octasaccharides (dp=8) eluted with 0.4M NaCl (see Fig. 4C). Only with dp10, and more substantially with the dp12/14 fraction, was higher affinity demonstrated such that 0.6M NaCl was required for elution (Figs. 4D and E respectively). Thus, oligosaccharides within the dp10-12 size range probably comprise the smallest high affinity HGF-binding oligosaccharides.

The disaccharide compositions of dp10 and dp12/14 oligosaccharides with different binding affinities were further analysed to more positively identify structural features correlating with HGF affinity. Oligosaccharides fractions recovered from the HGF column (Figs. 4D and 4E) were depolymerised by digesting using a combination of heparinases I, II and III and the resulting disaccharides were recovered by Bio-Gel P2 gel filtration chromatography. The fractions were then analysed and identified using a SAX-HPLC column calibrated with known disaccharide standards.

These analyses, of which the results are set out in Table 1, showed that although the total content of both $GlcNSO_3$ and IdoA(2S) increased slightly with increasing HGF affinity, a most dramatic correlation appeared in the content of 6-O-sulphates, in particular the $GlcNSO_3$(6S) residues. In the dp10 oligosaccharides the percentage of 6-O-sulphation was 18.4%, 29.8% and 50.6% in the low (0.2M), medium (0.4M) and high (0.6M) affinity fractions respectively. Similarly, in the dp12/14 oligosaccharides the corresponding medium and high affinity fractions contained 24.2% and 36.8% 6-O-sulphates respectively. As might be expected, these increases were only associated with N-sulphated disaccharides (primarily ΔUA-$GlcNSO_3$(6S) and ΔUA(2S)-$GlcNSO_3$(6S) disaccharides), and not with N-acetylated (6S) disaccharides whose abundance remained relatively constant. These two N-sulphated(6S) disaccharides would be expected to contain IdoA or IdoA(2S) respectively in the original oligosaccharides and to be located internally. The N-acetylated disaccharides would be expected to contain GlcA and could be derived from the reducing or non-reducing end (i.e. the sites of heparinase III cleavage), or might possibly be in an internal position where its environment may impart resistance to the enzyme.

It has thus been found not only that HS (and HSPG)

does bind to HGF under physiological conditions of pH and ionic strength, but Heparinase III digestion of HS will excise oligosaccharides which still retain most of the affinity for HGF. The smallest such oligosaccharides found to have the high HGF affinity are decasaccharides (dp10), although the minimum binding sequence (minimum core sequence which retains useful high affinity) could perhaps be shorter than this. Heparinase III-resistant sequences comprise mainly IdoA-containing disaccharides (with or without 2-sulphation) except for the non-reducing terminal, but non-sulphated IdoA residues appear to be important structural determinants of the HGF interaction. Clusters of IdoA(2S) residues are clearly not essential though there is some possibility that single residues may give some enhancement of binding. Heparinase III-resistant oligosaccharides will contain $GlcNSO_3$ residues internally, but the results of the desulphation experiment indicate that these are not absolutely essential for the interaction and make only a modest contribution to the binding process. In these oligosaccharides, $GlcNSO_3$ residues will, by necessity, be present in combination with IdoA(±2S) residues as the latter can only be introduced into the polysaccharide adjacent to existing $GlcNSO_3$ residues. In addition, it is clear from the disaccharide compositions of HS oligosaccharides with differing affinities for HGF that there is a strong correlation between the presence of 6-O-sulphation (of $GlcNSO_3$, although presumably GlcNAc would suffice) and high affinity. It is therefore deduced that HGF binding to HS requires repeat sequences of IdoA(±2S) - $GlcNSO_3$(6S) disaccharides occuring in oligosaccharides of $dp\geq 10$, as prepared from HS by partial depolymerisation with heparinase III. Moreover, although IdoA residues are considered to be essential IdoA(2S) residues may be regarded as optional for binding affinity.

It will accordingly be appreciated that oligosaccharide preparations with a specific HGF-binding

affinity have been obtained which are composed predominantly of oligosaccharide chains possessing one or more of the following features:

(a) a degree of polymerisation (dp) of at least 10 (preferably 10, 12 or 14, but not greater than 20);

(b) heparinase III resistance;

(c) HGF-binding affinity not destroyed by heparinase I;

(d) a relatively high proportion of 6-O-sulphated hexosamines;

(e) a structure that includes (preferably internally) repeat sequences (not necessarily all arranged contiguously) of IdoA(±2S)-$GlcNSO_3$(6S), possibly interrupted by occasional GlcNAc(±6S) components;

(f) an IdoA(2S) content, if any, which is less than the unsulphated IdoA content;

(g) a $GlcNSO_3$(6S) content which is greater than 24%, for example about 30% or preferably greater, up to say about 50% or more.

In practice, to produce the HGF-binding oligosaccharide products or preparations of the present invention, the same basic techniques can be used as described above in connection with the background experimental work. Thus, using a purified heparan sulphate as a starting material, this can be partially depolymerised by treatment with heparinase III (or other equivalent selective scission reagent) and subjected to affinity chromatography using an HGF-affinity matrix or substrate and eluting under a salt gradient, then selectively collecting fractions eluting at the higher salt concentrations to recover the material having the highest HGF-binding affinity, thereby providing a preparation of relatively short oligosaccharides which is substantially homogeneous with respect to HGF-binding affinity. By combining the affinity chromatography with a preceding and/or subsequent stage of gel filtration size exclusion chromatography and selecting fractions corresponding to a particular size or sizes, preferably dp$\geq$10 up to, say,

dp=20, and if desired carrying out further purifications by repeating these stages and/or using other purification methods such as SAX HPLC chromatography or gradient PAGE for example, well defined and purified preparations of the oligosaccharide products can be obtained which are substantially homogeneous both with respect to HGF-binding affinity and oligosaccharide chain size.

It is, however, also envisaged that sources other than heparan sulphate (or HSPG's), even for example heparin using an appropriate selective scission reagent for depolymerisation, may be used. Moreover, it may be possible to prepare equivalent HGF-binding oligosaccharides synthetically.

Since the oligosaccharides or preparations thereof in accordance with the invention can have a well defined composition and are readily capable of further purification if necessary, and considering also their relatively small sizes and specific HGF growth factor binding affinity, they can be very well suited for pharmaceutical use to exploit a potential in the field of medicine, e.g. as hepatocyte growth factor inhibitors or activators and mobilising agents. Accordingly, they are expected to have valuable applications as therapeutic drugs, particularly for controlling or regulating the activity of HGF. This may arise for example where there is a need to control or modulate HGF-activity dependent cell growth and proliferation or migration in clinical treatment of various conditions. For these purposes, the oligosaccharide products (or pharmaceutically-acceptable salts thereof) may be made up into pharmaceutical formulations as required, and such uses are also within the scope of the invention.

As will be seen, the invention provides a number of different aspects and, in general, it embraces all novel and inventive features and aspects, including novel

compounds, herein disclosed either explicitly or implicitly and either singly or in combination with one another. Moreover, the scope of the invention is not to be construed as being limited by the illustrative examples or by the terms and expressions used herein merely in a descriptive or explanatory sense.

TABLE I Disaccharide composition of HS oligosaccharides with differing affinities for HGF

Heparinase III-resistant oligosaccharides of size dp10 and dp12/14 were fractionated by affinity on HGF-Affigel. Oligosaccharide fractions eluted with 0.2M, 0.4M and 0.6M NaCl steps were recovered. These were depolymerised using a combination of heparinases and the resulting disaccharides were resolved by SAX-HPLC and quantified.

Disaccharide Structure	% Total Disaccharides				
	Dp10 oligosaccharides eluted with:			Dp12/14 oligosaccharides[a] eluted with:	
	0.2M	0.4M	0.6M	0.4M	0.6M
ΔHexA - GlcNAc	38.4	26.3	17.0	25.4	16.4
ΔHexA - GlcNAc(6-OSO_3)	9.1	11.6	11.1	10.9	11.7
ΔHexA - $GlcNSO_3$	28.3	21.6	14.9	23.2	19.8
ΔHexA - $GlcNSO_3$(6-OSO_3)	6.3	11.9	22.9	8.9	15.3
ΔHexA(2-OSO_3) - $GlcNSO_3$	14.9	22.3	12.4	27.1	24.8
ΔHexA(2-OSO_3) - $GlcNSO_3$(6-OSO_3)	3.0	6.3	16.6	4.4	9.8
unknown[b]	-	-	5.1	-	2.3
NSO_3/100 disaccharides	52.5	62.1	66.8	63.6	69.7
2-OSO_3/100 disaccharides	17.9	28.6	29.0	31.5	34.6
6-OSO_3/100 disaccharides	18.4	29.8	50.6	24.2	36.8

[a] There was insufficient of the 0.2M fraction for analysis

[b] From its elution position this is probably a disulphated disaccharide species

CLAIMS

1. An oligosaccharide preparation obtainable from partially depolymerised heparan sulphate (HS) or other natural heparin type material as a fraction thereof, characterised in that it consists essentially of oligosaccharide chains which have a specific binding affinity for hepatocyte growth factor (HGF) and which are composed of a sequence of at least three disaccharide units (dp≥6) that includes at least two disaccharide units containing an L-iduronic acid residue IdoA(±2S) and an N-sulphated D-glucosamine residue $GlcNSO_3$(±6S).

2. An oligosaccharide preparation comprising heparan sulphate (HS) fragments which have a specific binding affinity for hepatocyte growth factor (HGF) and which are composed of oligosaccharide chains containing a sequence of at least three disaccharide units (dp≥6) that includes at least two disaccharide units containing an L-iduronic acid residue IdoA(±2S) and an N-sulphated D-glucosamine residue $GlcNSO_3$(±6S).

3. An oligosaccharide preparation as claimed in Claim 1 or 2 in which said disaccharide units containing the IdoA(±2S) and $GlcNSO_3$(±6S) residues are disposed in between the terminal sugar residues of the oligosaccharide chains.

4. An oligosaccharide preparation as claimed in any of the preceding claims in which one or more of said at least two disaccharide units of the oligosaccharide chains is IdoA–α1,4–$GlcNSO_3$(6S).

5. An oligosaccharide preparation as claimed in any of the preceding claims further characterised in that the HGF-binding affinity is not completely destroyed by treatment under depolymerising conditions with heparinase I.

6. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that at least the majority of the oligosaccharide chains each have substantially the same length as a result of carrying out a size fractionation separation procedure.

7. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that at least the majority of the oligosaccharide chains each have a degree of polymerisation (dp) of 10 or more.

8. An oligosaccharide preparation as claimed in any of the preceding claims in which said oligosaccharide chains consist of a sequence of not more than ten disaccharide units in total.

9. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that at least the majority of the oligosaccharide chains each have a degree of polymerisation (dp) of 12 or 14.

10. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that said oligosaccharide chains are substantially completely resistant to further depolymerisation upon treatment under enzymic depolymerising conditions with heparinase III (heparitinase I).

11. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that the IdoA(2S) content, if any, of said oligosaccharide chains is less than the unsulphated IdoA content thereof.

12. An oligosaccharide preparation as claimed in any of the preceding claims further characterised in that the oligosaccharide chains contain a relatively high proportion of 6-O-sulphated hexosamines compared to oligosaccharide chains of unmodified native heparan

sulphate,

13. An oligosaccharide preparation as claimed in Claim 12, further characterised in that the $GlcNSO_3(6S)$ content of the oligosaccharide chains, i.e. number of residues per 100 disaccharides, is at least 30%.

14. An oligosaccharide preparation as claimed in Claim 12, further characterised in that the 6-O-sulphated hexosamine content of the oligosaccharide chains, i.e. number of residues per 100 disaccharides, is 50% or more.

15. An oligosaccharide preparation as claimed in any of the preceding claims in which the content of glucosamine residues in the oligosaccharide chains which are O-sulphated at C6 is greater than 24%.

16. An oligosaccharide preparation as claimed in Claim 15 in which the content of glucosamine residues in the oligosaccharide chains which are O-sulphated at C6 is about 35% or greater.

17. An oligosaccharide preparation as claimed in any of the preceding claims, further characterised in that the structure of the oligosaccharide chains includes internal repeat sequences of IdoA(±2S) and $GlcNSO_3(6S)$ interrupted by occasional GlcNAc(±6S) components.

18. An oligosaccharide preparation as claimed in any of the preceding claims in which substantially all said oligosaccharide chains consist of a sequence of six or seven disaccharide units in all.

19. An oligosaccharide preparation as claimed in any of the preceding claims further characterised in that it is obtainable from heparan sulphate (HS) of human fibroblast heparan sulphate proteoglycan (HSPG) by enzymic partial depolymerisation to the fullest extent with heparinase III

(heparitinase I) followed by size fractionation, using for example gel filtration size exclusion chromatography, followed by, in respect of a selected fraction or fractions recovered from the size fractionating stage, affinity chromatography using an HGF growth factor as the immobilised ligand in order to separate out HGF-binding fragments, and then eluting selectively over a range of salt concentrations under a salt gradient to fractionate said fragments in respect of HGF binding affinity, followed by recovering the most strongly bound fragments and, optionally, further purifying the recovered product by carrying out at least one additional step of size fractionation and selection of recovered product.

20. An oligosaccharide preparation made up of oligosaccharide chains having a specific binding affinity for human hepatocyte growth factor (HGF), characterised in that

(a) it is composed predominantly of a molecular species:

$$X-\left[-Y-\right]_n-Z$$

in which

X is ∩HexA-$GlcNSO_3$
Y is IdoA(±2S)-GlcR(±6S),
Z is IdoA-GlcR
where R is NSO_3 or NAc, and
n is in the range 1 to 5

with the proviso that when n is three or more then at least for the majority of said molecular species two or more of the $GlcR_3$ residues in Y are N-sulphated glycosamines sulphated at C-6, i.e. $GlcNSO_3$(6S);

(b) it is obtainable by a process comprising the steps of digesting a heparan sulphate with heparinase III (heparitinase I) so as to bring about partial depolymerisation thereof to the

fullest extent, followed by size fractionating the oligosaccharide mixture produced using for example gel filtration size exclusion chromatography, collecting a fraction or fractions containing oligosaccharide chains having a particular size selected within the range of 10 to 20 monosaccharide residues, then subjecting said selected fraction or fractions to affinity chromatography using an immobilised HGF ligand and recovering the more strongly HGF-binding constituents by eluting under a salt gradient over a range of salt concentrations and collecting a selected fraction or fractions containing the bound material which desorbs only at the highest salt concentrations.

21. An oligosaccharide preparation as claimed in Claim 20, wherein Y is primarily IdoA-GlcNSO$_3$(±6S).

22. An oligosaccharide preparation as claimed in Claim 20 or 21, wherein n is the range 3 - 5.

23. An oligosaccharide preparation as claimed in Claim 22 wherein said molecular species consists of a total of six or seven disaccharide units in all.

24. An oligosaccharide preparation as claimed in any of Claims 20 to 23 in which the content of glucosamine residues having a 6-O-sulphate group is greater than 24%.

25. An oligosaccharide preparation having a specific binding affinity for hepatocyte growth factors (HGF's) and substantially all composed of oligosaccharide chains which are twelve or fourteen monosaccharide residues in length and which contain an internal sequence comprising at least 2 disaccharide units each consisting of an IdoA residue linked to a GlcNSO$_3$(±6S) residue, with more than 20% of the glucosamine residues (terminal or internal) being 6-O-

sulphated.

26. An oligosaccharide preparation as claimed in Claim 25 wherein substantially all the oligosaccharide chains have the following sequence

$$\cap GlcA\text{-}GlcNSO_3\text{-}[IdoA\text{-}GlcNSO_3(\pm 6S)]_n\text{-}IdoA\text{-}GlcR$$

where R is NSO_3 or NAc, and n is 4 or 5.

27. A method of isolating from heparan sulphate derived from heparan sulphate proteoglycan of mammalian cells low molecular weight oligosaccharides in a purified and relatively homogeneous state which have a specific binding affinity for hepatocyte growth factor, said method comprising the steps of

(a) preparing an affinity chromatographic matrix or substrate incorporating a sample of hepatocyte growth factor (HGF) as the affinity ligand immobilised thereon;

(b) treating said heparan sulphate with a selective scission reagent so as to cleave the polysaccharide chains thereof selectively in regions of relatively low sulphation;

(c) subjecting the product of step (b) to size fractionation, for example by gel filtration size exclusion chromatography, and collecting selectively therefrom fractions that appear to contain oligosaccharides composed of less than ten disaccharide units,

(d) contacting the affinity chromatographic matrix or substrate from step (a) with a selected fraction, or set of fractions, from step (c) containing a specific number of disaccharide units in the range of five to seven in order to extract from the latter and retain on said matrix or substrate size selected oligo-saccharide fragments of the heparan sulphate glycosaminoglycan that have at least some binding affinity for the immobilised HGF;

(e) eluting the affinity chromatographic matrix or substrate using a progressively increasing salt concentration or gradient in the eluant;

(f) collecting the fraction or set of fractions containing oligosaccharide fragments eluting in selected highest ranges of eluant salt concentration; and optionally,

(g) further purifying the product of the selected fraction, or set of fractions, from step (f) by selectively repeating step (c) using said selected fraction or set of fractions collected in step (f) instead of the reaction mixture obtained from step (b), and optionally also repeating steps (d), (e) and (f).

28. A method as claimed in Claim 27 in which the selective scission reagent is heparinase III (heparitinase I) and the heparan sulphate is partially depolymerised to the fullest extent by digesting therewith until cleavage of the heparitase III sensitive linkages is complete.

29. A method as claimed in Claim 27 to 28, wherein the fractions collected from the size fractionation stage are those that appear to contain oligosaccharides composed of six or seven disaccharide units.

30. An oligosaccharide preparation as claimed in any one of Claims 1 to 26 for therapeutic use as an active HGF-activity modulating agent for controlling or reducing cell growth, proliferation or migration in treating mammals in need of such treatment.

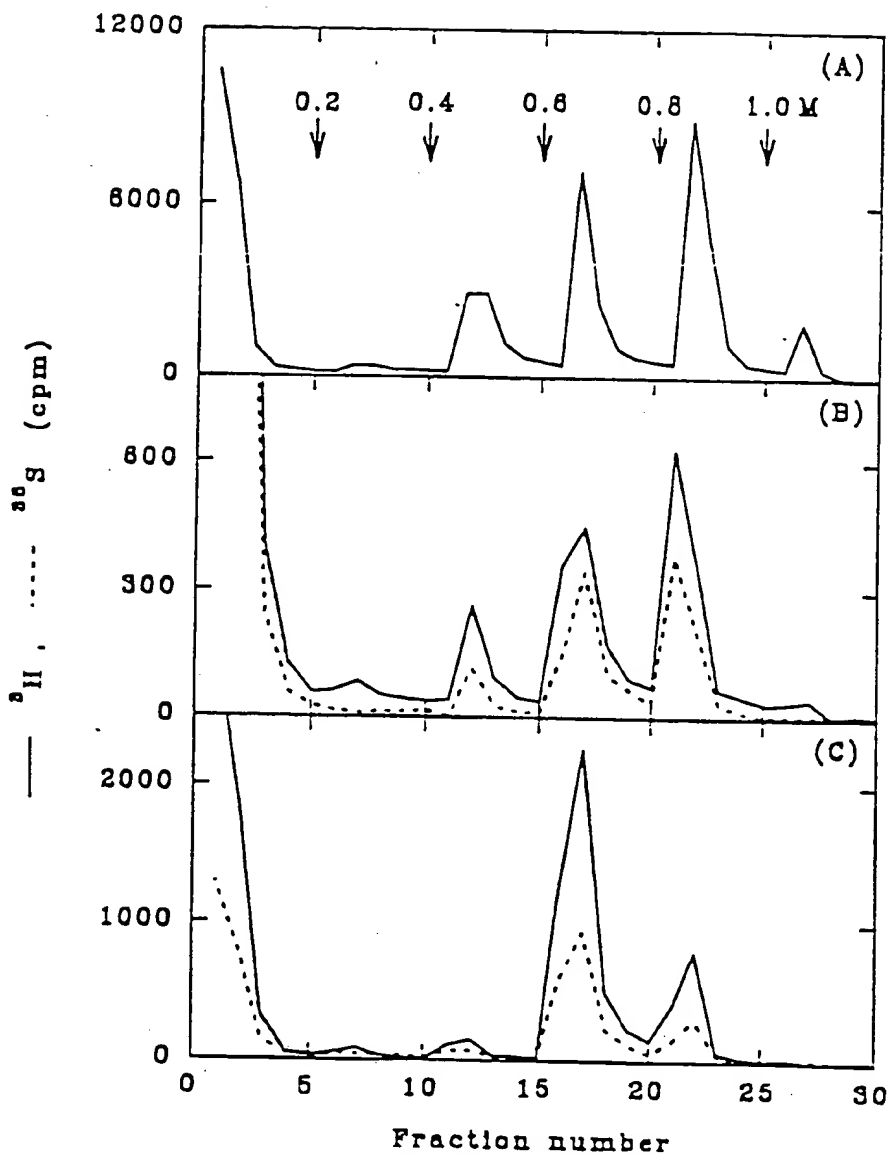
31. A pharmaceutical formulation or composition for medical use comprising a therapeutically effective non-toxic amount of an HGF-activity modulating agent comprising an oligosaccharide preparation as claimed in any of Claims 1 to 26 or pharmaceutically acceptable salts thereof, together with a pharmaceutically acceptable

carrier or vehicle.

32. An oligosaccharide preparation having a specific binding affinity for hepatocyte growth factors (HGF's), consisting essentially of linear oligosaccharide chains which are substantially homogeneous with respect to HGF binding affinity and which contain a sequence of less than ten disaccharide units including, intermediate its terminal residues, a plurality of disaccharide units each composed of an N-sulphated glucosamine residue (±6S) and an unsulphated iduronic acid residue.

33. A pharmaceutical composition or formulation for use in controlling the activity of hepatocyte growth factors in mammals, said composition or forumulation comprising a therapeutically useful amount of an essentially pure oligosaccharide preparation as claimed in Claim 32.

FIG. 1.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Fraction number

FIG. 2.

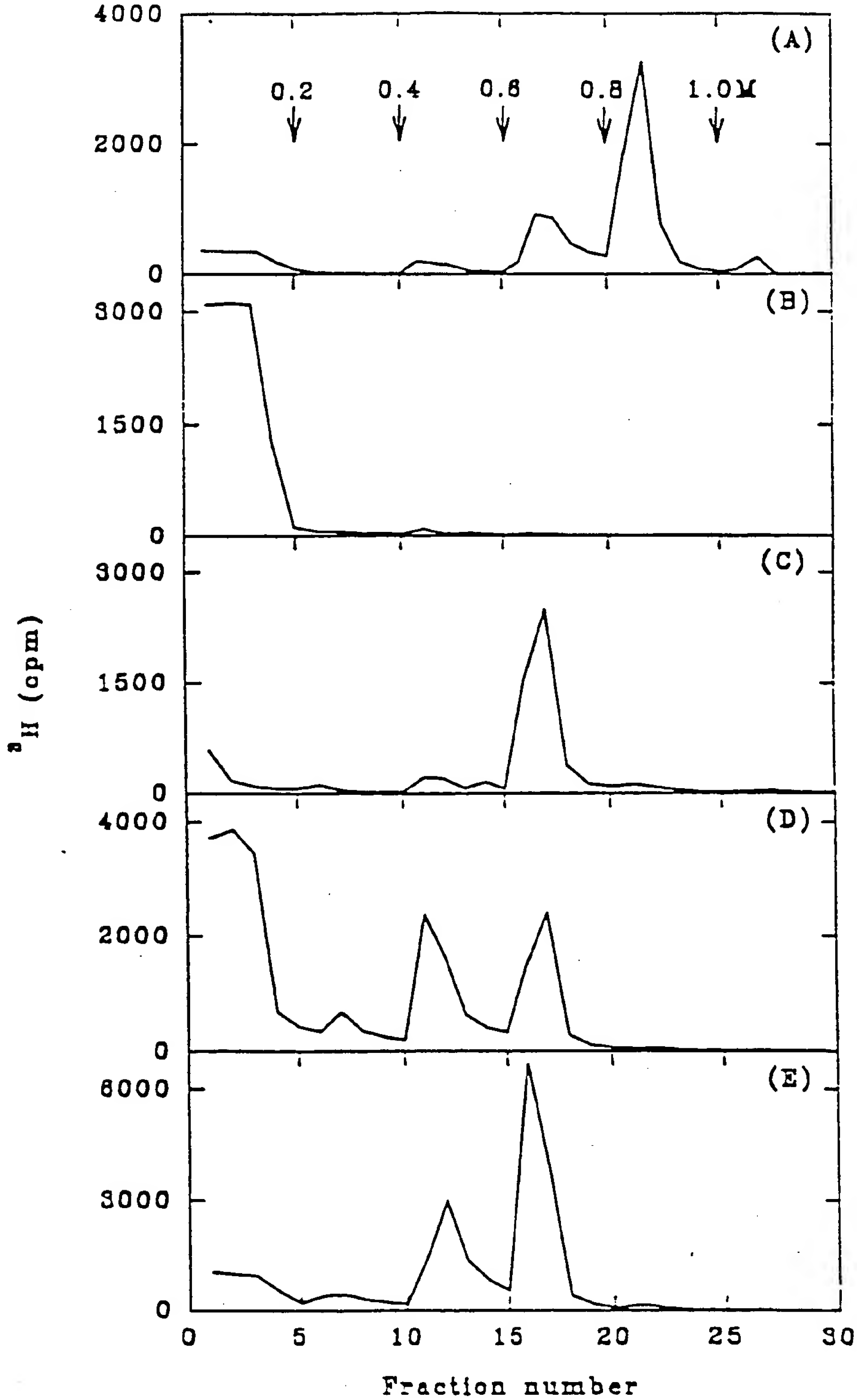
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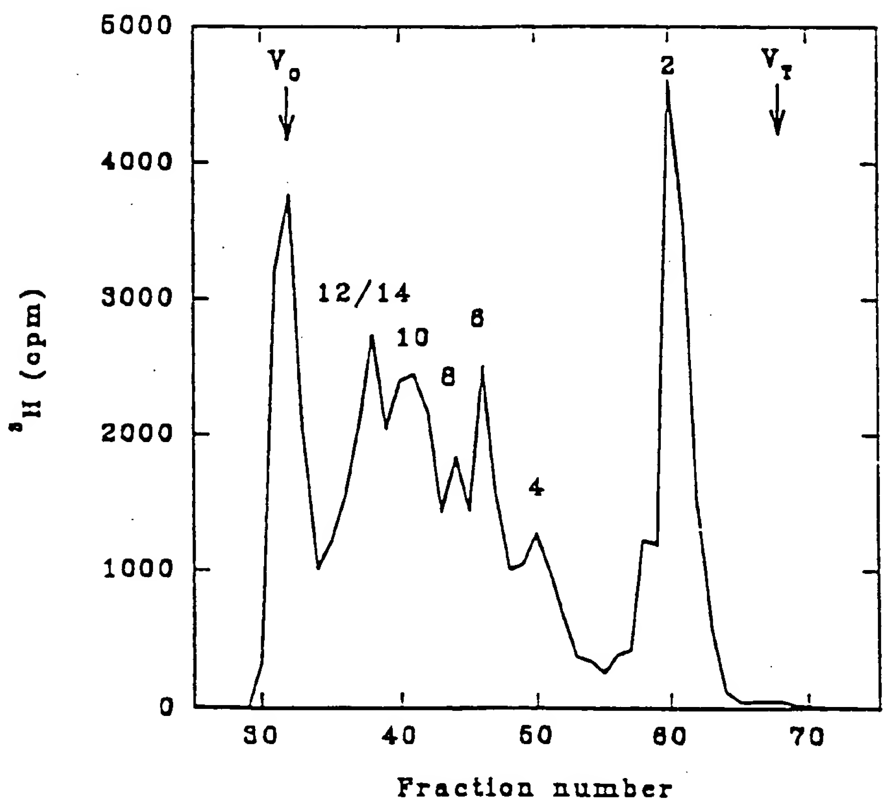
FIG. 3.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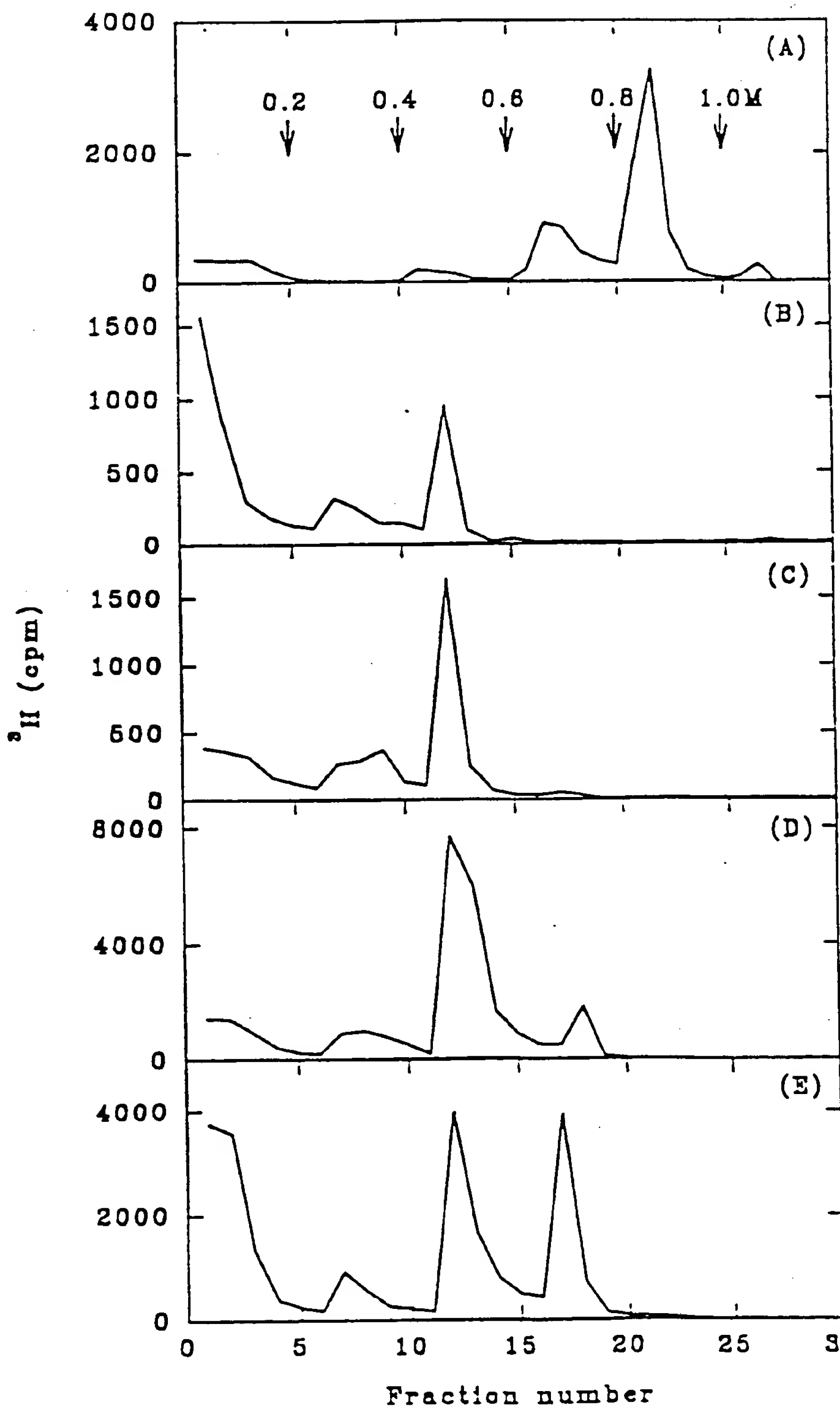
Fraction number

FIG.4.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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00615

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C08B37/10 A61K31/725

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 244 298 (SANOFI) 4 November 1987 see page 1, line 1 - line 7 see page 3, line 4 - line 15 see page 4, line 1 - line 18 see page 5, line 3 - line 17 see page 8, line 23 - line 33 see page 10, line 25 - page 11, line 5 see page 16, line 6 - line 10 see page 28, line 21 - line 27 see page 29, line 6 - page 30, line 5	1,2
Y	see claims ---	19,20, 27-31
Y	EP,A,0 517 182 (MITSUBISHI KASEI CORPORATION) 9 December 1992 see page 3, line 32 - line 34 see page 6, line 34 - line 37 see claims ---	19,20, 27-31

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1994	- 4. 08. 94

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 509 517 (SEIKAGAKU KOGYO KABUSHIKI KAISHA) 21 October 1992 see the whole document	1,2,7,8
A		19,20,27
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